

Method of measuring the luminescence emitted in a luminescent assay

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5 The invention relates to a method of measuring the luminescence emitted in a luminescent assay, which makes it possible to correct certain perturbations due to the measuring medium.

At the present time, immunoassays are widely used for the qualitative and quantitative analysis of compounds in biological fluids.

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Among the techniques in existence, fluorimetric assays have become increasingly important.

In fact, they have a number of advantages, including the sensitivity and rapidity of the measurement, the stability and safety of the reagents labeled with fluorescent compounds, and the relatively low cost.

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It is known that detection methods which use fluorescence are intrinsically very sensitive and could permit lower detection limits than those achieved by immunoassays which use radiolabeled reagents, in particular if modulatable laser light sources are used (I. Wieder, Immunofluorescence and related staining techniques, 1978, Elsevier).

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Numerous fluorescent molecules usable as tracers in assays of this type have previously been described; among these, rare earth complexes, which possess valuable properties, may be mentioned in particular.

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"Tracer" is understood as meaning either a luminescent molecule emitting a direct luminescence, or a luminescent molecule capable of inducing a luminescent emission, it being possible for said molecule to be coupled with one of the reagents of the assay, and the emission of a direct or induced luminescence enabling the target analyte to be detected

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and/or determined.

The use of particular complexes, rare earth cryptates, is described for example in European patent applications 0 321 353, 0 180 492 and 0 232 348 or
5 international patent application WO 90/04791.

These rare earth cryptates have the advantage of being very stable in a saline protein medium, this property being particularly important in the case of homogeneous immunoassays.

10 The sensitivity of the measurement is nevertheless limited by different interference parameters, among which the following may be mentioned:

- the spectroscopic properties of the medium, and in particular its intrinsic fluorescence, which is
15 due especially to the interference emissions of the molecules present in the measuring medium and capable of being excited and of emitting at wavelengths close to those of the fluorescent tracer and/or with strong intensities; its absorption, which results in a loss of
20 exciting light; and its light diffusion properties when the measuring medium is not clear;

- the quenching of the emitted fluorescence by inhibitors present in the medium; and

- the composition of the equipment, and
25 especially the interference reflections caused by the equipment.

Together these interferences considerably affect the sensitivity and reproducibility of the measurement.

30 Some of these problems have already been solved by a variety of techniques.

In particular, the time-resolved methods of measuring fluorescence enable the problem of interference emissions (background) to be partially
35 overcome. The principle of these methods consists in measuring the fluorescence emitted by a tracer molecule

fluorimeter which makes it possible to measure, in parallel with the fluorescence of the sample, the transmission of the sample and the fluctuations in excitation energy, in order to correct the measured
 5 fluorescence. This system requires a particular measuring cell which allows the exciting beam to pass through, since the transmission measurement has to be made in line with the exciting beam.

Other systems employ a system for splitting the
 10 exciting beam, as described for example in European patent applications 174 722 and 241 268.

The invention therefore relates to a method of measuring the luminescence emitted in a luminescent assay, which comprises employing at least one
 15 luminescent tracer compound and a luminescent compound used as an internal reference, which, when exposed to the same excitation wavelength, are capable of emitting at different wavelengths, λ_2 and λ_1 respectively, either by direct luminescence or by the induction of a
 20 luminescent emission, the luminescence of the reference compound reflecting the optical quality of the medium, and correcting the measurement of the luminescence emitted by the tracer compound at wavelength λ_2 on the basis of the measurement of the luminescence emitted by
 25 the reference compound at wavelength λ_1 .

"Reference compound" is understood as meaning either a luminescent molecule emitting a direct luminescence, or a luminescent molecule capable of inducing a luminescent emission, said emission of a
 30 direct or indirect luminescence not being perturbed by the reagent system of the assay.

The luminescent compounds which can be used in the method of measurement according to the invention can emit directly either at their emission wavelength
 35 or at another wavelength, as for example in the case of a spectral shift associated with the reagent system of

the assay.

If appropriate, the luminescent compounds which can be used in the method of measurement according to the invention can emit indirectly by inducing a luminescent emission, especially as in the case of homogeneous energy transfer methods.

Advantageously, the luminescent emissions at wavelengths λ_2 and λ_1 are detected simultaneously.

The method according to the invention, which uses a single exciting beam, permits easy and reliable measurement of the luminescence emitted in a luminescent assay without the need for complex equipment, by eliminating the perturbations due to the spectroscopic properties of the assay medium.

Advantageously, the emission wavelengths of the luminescent tracer compound and luminescent reference compound, λ_2 and λ_1 , will be different but preferably similar (the difference being less than or equal to 100 nm, for example) so that the perturbation of the luminescent emission which is due to the absorption of the medium is produced in the same manner as regards the emission of the tracer compound and that of the reference compound.

It is pointed out that, advantageously, the method according to the present invention does not require the assay sample to be placed in a special measuring cell.

The luminescent emission of the reference compound at wavelength λ_1 enables the measurement made at wavelength λ_2 to be corrected. The correction can be effected for example by dividing the latter measurement by the measurement made at wavelength λ_1 . Other means of correction can be used, for example a correction method, built into the apparatus, which consists in fixing a counting rate on the channel measuring the luminescent emission of the reference compound at

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wavelength λ_1 . When this rate is reached, the end of the measurement on the channel measuring the luminescent emission at wavelength λ_2 is triggered. The value obtained on this channel is thereby corrected
5 directly.

Other correction methods known to those skilled in the art can also be used.

According to a preferred feature, the invention relates to a method of measuring the fluorescence emitted in a fluorometric assay, which comprises
10 employing at least one fluorescent tracer compound and a fluorescent compound used as an internal reference, which, when exposed to the same excitation wavelength, are capable of emitting at different wavelengths, λ_2 and λ_1 respectively, either by direct fluorescence or
15 by the induction of a fluorescent emission, the fluorescence of the reference compound reflecting the optical quality of the medium, and the measurement of the fluorescence emitted by the tracer compound then
20 being corrected on the basis of the measurement of the fluorescence emitted by the reference compound.

The method according to the invention makes it possible to achieve a measuring sensitivity of the order of picomol/liter, whereas the phenomena referred
25 to above usually limit the sensitivity of the assay, especially in the case of homogeneous assays in a serum medium, to analyte concentrations of the order of micromol/liter.

According to an advantageous feature of the invention, the luminescent tracer compound and the
30 reference compound are one and the same compound.

This first variant of the method according to the invention preferably applies when using a homogeneous luminescent method of detecting and/or
35 determining an analyte in a medium in which it may be present, where the measurement of the emitted

in:

1) ~~adding, to said medium~~ containing the target analyte, a first reagent made up of at least one receptor for said analyte, coupled with a luminescent donor compound.

2) adding a second reagent made up of one or more other receptors for said analyte, said second reagent being coupled with a luminescent acceptor compound,

3) incubating said medium after the addition of each reagent or after the addition of both reagents,

4) exciting the resulting medium at the excitation wavelength of the luminescent donor compound, and

5) measuring the signal of the luminescent donor compound at a wavelength λ_1 , this measurement serving as a reference, and the signal resulting from the energy transfer at a different wavelength λ_2 .

According to an advantageous feature, the first and second reagents used in the above-indicated luminescent methods of detecting and/or determining an analyte are added simultaneously to the medium containing the target analyte.

According to another feature of the invention, this variant of the method can be used in a homogeneous luminescent method of detecting and/or determining an analyte in a medium in which it may be present, with the aid of a competitive method consisting in:

1) adding, to said medium containing the target analyte, a first reagent made up of at least one receptor for said analyte, coupled with a luminescent donor compound,

2) adding a second reagent made up of the analyte coupled with a luminescent acceptor compound,

3) incubating said medium after the addition of each reagent or after the addition of both reagents,

4) exciting the resulting medium at the excitation wavelength of the luminescent donor compound, and

5) measuring the signal of the luminescent donor compound at a wavelength λ_1 , this measurement serving as a reference, and the signal resulting from the energy transfer at a different wavelength λ_2 .

This variant of the method can also advantageously be used in a homogeneous luminescent method of detecting and/or determining an analyte in a medium in which it may be present, with the aid of a competitive method consisting in:

1) adding, to said medium containing the target analyte, a first reagent made up of at least one receptor for said analyte, coupled with a luminescent acceptor compound,

2) adding a second reagent made up of the analyte coupled with a luminescent donor compound,

3) incubating said medium after the addition of each reagent or after the addition of both reagents,

4) exciting the resulting medium at the excitation wavelength of the luminescent donor compound, and

5) measuring the signal of the luminescent donor compound at a wavelength λ_1 , this measurement serving as a reference, and the signal resulting from the energy transfer at a different wavelength λ_2 .

In another advantageous use of the method of the invention, at least one of the receptors for the analyte is bound to a solid support.

According to another advantageous feature, the luminescent tracer compound and the reference compound are 2 different compounds excitable at the same wavelength, the reference compound emitting at a different wavelength from that used to measure the quantity of analyte.

This second variant of the method of the invention is preferred in particular in the use of a homogeneous luminescent method of detecting and/or determining an analyte in a medium in which it may be present, said method using a reagent coupled with a luminescent tracer compound and a reagent coupled with a heavy atom, or moieties containing a heavy atom, capable of modulating the signal of the luminescent tracer compound. An assay method of this type is described in European patent application 232 348.

In this case, in addition to the luminescent tracer compound coupled with the receptor for the analyte and emitting at a wavelength λ_2 , another, free luminescent compound, serving as a reference and emitting at a wavelength λ_1 , will be used whose signal is not modulated by the heavy atom effect and reflects the optical properties of the measuring medium, these 2 compounds being excited at the same wavelength.

Advantageously, this second variant of the method according to the invention is used in a homogeneous luminescent method of detecting and/or determining an analyte in a medium in which it may be present, consisting in:

- 1) adding to said medium a first reagent made up of a receptor for said analyte,
- 2) adding a second reagent selected from the analyte or at least one of its receptors, one of the two reagents being coupled with a luminescent tracer compound and the other reagent containing a heavy atom or moieties containing a heavy atom, as well as a luminescent compound serving as an internal reference,
- 3) incubating the resulting medium either after the addition of each reagent or after the addition of both reagents,
- 4) exciting the resulting medium, and
- 5) measuring on the one hand the signal emitted

by the luminescent tracer compound, said signal being modulated by the heavy atom effect at a wavelength λ_2 , and on the other hand the signal emitted by the reference compound at a wavelength λ_1 .

5 This second variant of the method, in which the luminescent tracer compound and the reference compound are two different compounds, can also advantageously be used in homogeneous methods of detecting and/or determining an analyte in a medium in which it may be present, with the aid of excess or competitive methods
10 involving a donor/acceptor system, such as those described above for the first variant of the method of the invention.

15 Nevertheless, in the case of the second variant, a luminescent compound serving as an internal reference should be added during one of the reagent addition steps.

20 The signal of the luminescent reference compound will then be measured at a wavelength λ_1 and the signal resulting from the energy transfer will be measured at a wavelength λ_2 , it then being possible for this measurement to be corrected on the basis of the measurement made at λ_1 .

 In the present description:

- 25 - "analyte" defines any substance or group of analogous substances to be detected and/or determined;
 - "receptor" defines any substance capable of binding specifically to a site on said analyte;
 - "heavy atom" defines an atom of high atomic
30 number whose presence in the proximity of a fluorescent molecule is capable of inducing an increase in the spin-orbit coupling of the latter; examples which may be mentioned of appropriate heavy atoms are especially halogen atoms, mercury, thallium, lead and silver;
35 - "moiety containing at least one heavy atom" defines any chemical substance which naturally contains

at least one heavy atom or to which at least one heavy atom can be bound.

According to a preferred feature, the measuring medium is a biological medium such as a serum medium.

5 Fluorescent compounds will advantageously be used as luminescent reference compounds and/or luminescent compounds usable as tracer compounds.

10 In particular, a fluorescent compound such as a rare earth chelate or cryptate will advantageously be used, especially a terbium, europium, dysprosium, samarium or neodymium chelate or cryptate. A terbium or europium cryptate will preferably be used.

15 In the fluorescent methods of detection and/or determination using the method of measurement of the invention, a rare earth cryptate described in European patent applications 180 492 and 321 353 will advantageously be chosen.

20 The terbium cryptate Tb trisbipyridine or the europium cryptate Eu trisbipyridine, as described in European patent application 180 492, or the cryptates Eu trisbipyridinediamine and Tb trisbipyridinediamine, described in European patent application 321 353, will preferably be used.

25 According to an advantageous feature, the fluorescent donor compound is a europium cryptate and the fluorescent acceptor compound is selected from allophycocyanin, allophycocyanin B, phycocyanin C or phycocyanin R.

30 It is also possible to use a phosphorescent compound, such as eosin or erythrosin, as the luminescent reference compound or donor tracer compound. In this case, it will be advantageous to use a fluorescent acceptor compound selected from chlorophylls such as those mentioned in European patent
35 applications 71 991 and 314 406, or porphyrins such as those mentioned in European patent application 71 991,

or else phthalocyanins such as those of international patent application WO 88 04777.

In the case of an assay in a liquid medium using phosphorescent donor compounds, the result will be read either from a solid support or by adding oxygen-capturing molecules to the measuring medium, these techniques being known to those skilled in the art.

Chlorophylls and phthalocyanins can also be used as fluorescent acceptor compounds, a europium cryptate or chelate being used as the donor compound.

According to a preferred feature, the luminescent tracer compound and/or the luminescent reference compound have a lifetime of more than one microsecond.

A modulatable light source, such as those described in Lakowicz, Principles of fluorescent spectroscopy, Plenum Press, New York, 1983, pp. 96-100, will advantageously be used as the light source permitting the excitation of the luminescent tracer and reference compounds.

The method of measurement of the invention has an important application in particular in fluorescent immunoassays, in both the so-called competitive and excess assay methods, which have been described in the prior art (Landon, Ann. Clin. Biochem., 1981, 18, 253, and E. SOINI et al., Clin. Chem., 1979, 25, 353).

In particular, the method of measurement of the invention can advantageously be used in immunoassays in a serum medium.

A further feature of the invention relates to a device for carrying out the method of measurement according to the invention.

This device comprises an exciting light source, means for collecting the light beam emitted following said excitation, and means for measuring the

luminescence at two different wavelengths.

Advantageously, this device also comprises means for splitting the beam emitted following excitation.

5 Such a device is illustrated by way of example in Figure 1.

Figure 1 shows:

- an exciting light source 1;
- a lens 2, which focuses the exciting beam
10 towards a filter 3 for selecting the desired excitation wavelength;
- a dichroic filter 4 arranged at 45°, for reflecting the ultraviolet rays and transmitting the visible light, which reflects the exciting beam on to a
15 lens 5, the latter focusing it on to the microplate 6 containing the samples;
- a lens 7, which, in combination with the lens 5, collects the luminescent emission resulting from the excitation and projects it on to a dichroic filter 8,
20 which splits the emitted light beam; and
- filters 9 and 10, which select the signals emitted by the reference compound and tracer compound before they arrive at photomultipliers 11 and 12.

The invention will be understood more clearly
25 with the aid of the Examples below, which in no way imply a limitation.

EXAMPLE 1:

30 Correction of fluorescence at 665 nm by the detection of an added terbium cryptate (trisbipyridine-diamine (Tb^{3+})) in a homogeneous fluorescent immunoassay of prolactin

This Example is carried out on the detection of a standard antigen range and on the assay of 5 serum
35 samples of unknown prolactin concentration.

METHODOLOGY:

This assay uses as the fluorescent donor compound the europium cryptate trisbipyridinediamine (Eu^{3+}) (tracer) and as the reference compound the terbium cryptate trisbipyridinediamine (Tb^{3+}), both prepared as described in European patent application 321 353 (Examples 3 and 4).

Allophycocyanin (Cyanotech, USA) is used as the fluorescent acceptor compound.

Anti-prolactin E_1 and 3D3 monoclonal antibodies (CIS bio international, France), recognizing 2 different epitopes of prolactin, are used. The preparation of the antibodies labeled with the europium cryptate trisbipyridinediamine (Eu^{3+}) or with allophycocyanin is described below:

The following abbreviations are used:

APC	= allophycocyanin
DTT	= dithiothreitol
Eu TBP	= europium cryptate trisbipyridinediamine (Eu^{3+})
HSA	= human serum albumin
IgG	= immunoglobulin G
SPDP	= N-succinimidyl 3-(2-pyridyldithio)-propionate
Sulfo-SMCC	= sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate

1) PREPARATION OF IgG E_1 /APC

a) Activation of APC with sulfo-SMCC

APC (3 mg), supplied commercially in precipitated form in a 60% solution of ammonium sulfate, is centrifuged. After removal of the supernatant, the residue is taken up in 250 μl of 100 mM phosphate buffer, pH 7.0, and then passed through a 0.8 μm filter to remove any suspended particles.

The filtrate is purified by exclusion

blocked by the addition of a 100 mM solution of N-methylmaleimide (Sigma, USA), having a final concentration of 20 mM, for one hour at room temperature.

5 The reaction medium is purified by gel filtration on a TSK G3000SW semipreparative column (Beckmann, USA) in 100 mM phosphate buffer, pH 7.0.

The concentrations of APC and IgG E₁ in the purified conjugate eluted in the first peak are determined by the absorptions at 280 nm and 650 nm according to the following calculation:

$$[\text{APC}]_{\text{mol/l}} = A_{650\text{nm}}/710,000$$

$$[\text{IgG}]_{\text{mol/l}} = (A_{280\text{nm}} - A'_{280\text{nm}})/210,000$$

10 where A'280nm is the contribution of the APC-maleimide at this wavelength.

Human serum albumin (HSA) is added at a rate of 1 g/l to the conjugate, which is then divided up into aliquots and subsequently frozen at -20°C.

20 2) PREPARATION OF IgG 3D3/Eu TBP CONJUGATES

IgG 3D3-SH is prepared according to the protocol described above for IgG E₁, but with a molar ratio of 7.5 SPDP per IgG 3D3.

25 A 25 mM solution of sulfo-SMCC in 20 mM phosphate buffer, dimethylformamide 10% (v/v), pH 7.0, is added to 5 mg ($5 \cdot 10^{-6}$ mol) of Eu TBP in a proportion of 2.5 mol of activator per mol of Eu TBP.

After activation for 45 min at room temperature, the reaction medium is passed through a 30 0.8 µm filter to remove any precipitate formed. The unwanted reaction products (sulfo-SMCC, N-hydroxy-succinimide, (N-maleimidomethyl)carboxylic acid) are removed by ion exchange chromatography on a Mono Q column (Pharmacia, Sweden) in 20 mM phosphate buffer, 35 dimethylformamide 10% (v/v), pH 7.0, under NaCl shock. The concentration of Eu TBP-maleimide is determined at

In a manner similar to that described above, the maleimide groups are reacted with the thiol groups bound to the antibody, in molar proportions varying from 10 to 30 Eu TBP-maleimide per IgG 3D3-SH.

The characteristics of the conjugate are determined by its absorptions at 307 nm and 280 nm using the following values, taking into account the inherent absorption of the cryptate determined by the ratio $A_{307\text{nm}}/A_{280\text{nm}}$.

20 $\epsilon_{307\text{nm}} = 25,000 \text{ M}^{-1} \text{ cm}^{-1}$
 $A_{307\text{nm}}/A_{280\text{nm}}$: determined experimentally
 IgG 3D3-SH:

$$\epsilon_{280\text{nm}} = 210,000 \text{ M}^{-1} \text{ cm}^{-1}$$
$$\epsilon_{307\text{nm}} = 0 \text{ M}^{-1} \text{ cm}^{-1}$$

- 100 µl of standard (prolactin standards from the ELSA-PRL kit (CIS Bio international) in newborn calf serum) or 100 µl of assay sample (human serum),

35 - 50 μ l of anti-prolactin E₁ monoclonal
antibody labeled with allophycocyanin, at a

concentration of 7 $\mu\text{g/ml}$ of antibody in 100 mM phosphate buffer, NaF 150 mM, BSA 1 g/l, pH 7.0, and
- 50 μl of the terbium cryptate trisbipyridinediamine (Tb^{3+}), 10^{-7}M in 100 mM phosphate
5 buffer, NaF 150 mM, BSA 1 g/l, pH 7.0.

After incubation for one hour at 37°C , the fluorescence of each well is measured according to two protocols:

In a first protocol, the fluorescence at 665 nm
10 is detected by the time-resolved method with a measuring delay of 50 μs and an integration time of 400 μs . The measuring time is 1 s. The excitation is caused by a flashlamp pulsed at 1000 Hz. The excitation wavelength is centered by an interference filter at 307
15 nm, which is the absorption maximum of trisbipyridinediamine (Eu^{3+}) and trisbipyridinediamine (Tb^{3+}). The recorded intensity of fluorescence, which is a reflection of the energy transfer, is proportional to the concentration of prolactin antigen present in
20 the incubation medium.

The measurement is made by means of an ARCUS fluorimeter (LKB, Sweden) using an interference filter adapted to the emission of the fluorescent acceptor compound and the reference compound.

25 It is also possible to make the measurement in a single step using the prototype fluorimeter described in Example 2. In this case, a 96-well white microplate (Dynatech, USA) will be used for the measurement.

The standard curve $\text{FLUO}_{665\text{nm}} = f([\text{prolactin}])$
30 is plotted and the concentration of the 5 samples is measured.

The results are reported in Table I below:

TABLE I

	ELSA-PRL standard	[Prolactin] μ IU/ml
	STD ₀	0
5	STD ₁	165
	STD ₂	300
	STD ₃	920
	STD ₄	3100
	STD ₅	6500
10	Assay serum	[Prolactin] (FLUO ₆₆₅) μ IU/ml
	Sam ₁	141
	Sam ₂	951
	Sam ₃	113
15	Sam ₄	287
	Sam ₅	699

In a second protocol, the fluorescence at 665 nm is measured in the same way as described above. However, a second measurement of the same well is made at 545 nm after a second excitation, with the same temporal reading parameters (delay, integration time and measuring time). The fluorescent emission thus measured by the time-resolved method at 545 nm is characteristic of the emission of the trisbipyridinediamine (Tb^{3+}) used as the internal reference and reflects the optical properties of the assay medium.

The standard curve $FLUO_{665nm}/FLUO_{545nm} = f([prolactin])$ is plotted and the concentration of the 5 samples is measured.

The results are reported in Table II below:

TABLE II

ELSA-PRL standard		[Prolactin] μ IU/ml
5	STD ₀	0
	STD ₁	165
	STD ₂	300
	STD ₃	920
	STD ₄	3100
	STD ₅	6500
10		
Assay serum		[Prolactin] (FLUO ₆₆₅ /FLUO ₅₄₅) μ IU/ml
15	Sam1	580
	Sam2	428
	Sam3	417
	Sam4	71
	Sam5	2044

Finally, as a reference, the 5 samples are also assayed by the ELSA-PRL radioimmunometric assay kit (CIS bio international, France).

The comparisons of the results of assays performed on the five samples are collated in Table III below:

TABLE III

		[Prolactin] μ IU/ml		
		ELSA-PRL	FLUO ₆₆₅	FLUO ₆₆₅ /FLUO ₅₄₅
30	Sam1	612	141	580
	Sam2	395	951	428
	Sam3	425	113	417
	Sam4	59	287	71
	Sam5	1870	699	2044

These results show that the measurements of the

photomultiplier (HAMAMATSU R2949).

The photon counter used is an SR-400 (STANFORD RESEARCH SYSTEMS), whose operations and synchronization with the laser are controlled by a computer of the IBM PC-AT type via an RS 232 output. The pulses coming from the photomultiplier are recorded over a given time window (t_g) and after a given delay (t_d), provided they are above a discriminant level selected by the photon counter so as to optimize the signal-to-noise ratio of the photomultiplier.

An X-Y table, driven by the IBM PC-AT, uses step motors to set up the different positions of the measuring microplate, including the maneuvers of charging, positioning under the exciting beam, automatic sequential reading of the 96 wells, and exiting.

I. Variation of the signal of a cryptate conjugate at 620 nm as a function of the serum

The following are introduced into a 96-well white microplate (Dynatech, USA):

- 100 μ l of test serum, and
- 200 μ l of an anti-prolactin antibody/europium cryptate conjugate at a concentration of 0.5 μ g/ml of antibody in 100 mM phosphate buffer, pH 7.5, HSA 1 g/l, NaF 150 mM.

The europium cryptate used is trisbipyridinediamine (Eu^{3+}), the preparation of which is described in European patent application 321 353 (Examples 3 and 4).

The anti-prolactin antibody used is the 3D3 antibody (CIS bio international, France).

The 3D3 antibody/europium cryptate conjugate is prepared as described in Example 1.

After incubation for 1 hour at 37°C, the fluorescence is detected at 620 nm by the time-resolved

method with a measuring delay of 50 μ s and an integration time of 400 μ s. The measuring time is 1 s.

The optical density of the serum is measured at 310 nm with the aid of a Lambda 15 spectrophotometer from PERKIN ELMER (UK).

The results are reported in Table IV below:

TABLE IV

Serum	OD at 310 nm	Signal at 620 nm
1	0.66	25,693
2	3.3	7,754
3	0.33	33,156
4	0.90	24,959

These results show that the measured signal varies greatly as a function of the optical density of the serum.

Under these conditions, it is impossible to perform a reliable assay because the result depends not only on the quantity of analyte in the serum but also on the optical quality of the serum.

II. Correction of the signal of the acceptor

An immunoassay is then performed by successively introducing the following into the microplate:

- 100 μ l of serum,

- 100 μ l of anti-prolactin 3D3 monoclonal antibody/europium cryptate trisbipyridinediamine (Eu^{3+}) conjugate at a concentration of 0.5 μ g/ml of antibody in the above phosphate buffer, and

- 100 μ l of anti-prolactin E_1 monoclonal antibody/allophycocyanin conjugate at a concentration of 3.5 μ g/ml of antibody in the same buffer.

This E_1 antibody (CIS bio international,

France)/allophycocyanin (Cyanotech, USA) conjugate is prepared as described in Example 1.

The measurement is made at 665 nm and the measured value is corrected by dividing this value by the value of the fluorescence measured at 620 nm, which reflects the optical properties of the medium.

The values of concentrations determined with the aid of a calibration curve as indicated in Example 1, with or without correction, are compared with the values obtained by using the ELSA-PRL radioimmuno-metric assay kit (CIS bio international, France) as a reference.

The results are reported in Table V below, which shows the values of the prolactin concentrations, expressed in $\mu\text{U/ml}$, determined respectively by the ELSA-PRL assay (ELSA) and by measurement of the fluorescence at 665 nm without correction (FIA_{665}) and with correction ($\text{FIA}_{665/620}$), as well as the value of the fluorescence measured for the different samples at 620 nm (FLUO_{620}), expressed in counts per second.

TABLE V

	Sample no.	ELSA	$\text{FIA}_{665/620}$	FIA_{665}	FLUO_{620}
25	1	358	394	195	42,148
	2	530	592	91	28,272
	3	65	92	18	40,495
	4	153	178	94	43,363
	5	251	289	86.7	38,007
30	6	161	146	86.4	44,531
	7	179	189	77	40,873
	8	285	297	161	41,403
	9	310	330	106	35,214
	10	2744	3427	1470	31,670
35	11	196	153	15	36,551
	12	244	198	55	38,134

TABLE V (continuation)

	Sample no.	ELSA	FIA _{665/620}	FIA ₆₆₅	FLUO ₆₂₀
5	13	447	477	300	40,340
	14	251	234	350	58,615

These results confirm the variability of the measured signal as a function of the serum sample assayed.

In fact, given that the antibody/cryptate conjugate is present in excess in the assay, compared with the target analyte, the signal measured at 620 nm (which corresponds to the conjugate not forming part of the complex) should be constant.

Furthermore, it is pointed out that the values of prolactin concentrations determined from the measurement of the signal at 665 nm (FIA₆₆₅) do not correspond to the values obtained by the reference assay, whereas the values obtained by the corrected measurement (FIA_{665/620}) correlate well with the latter.

This correlation is illustrated by the curves of Figures 2 and 3, in which the abscissa shows the prolactin concentration determined by the ELSA-PRL test, in IU/ml, and the ordinate respectively shows the concentration determined by the corrected measurement FIA_{665/620} (Figure 2) or by the uncorrected measurement FIA₆₆₅ (Figure 3), expressed in IU/ml.

The results obtained on 97 sera show that the correlation is obtained only with correction of the signal measured at 665 nm.

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